Improved pharmaceutical impurity determination via comprehensive 2D-LC temperature-responsive x reversed phase liquid chromatography.

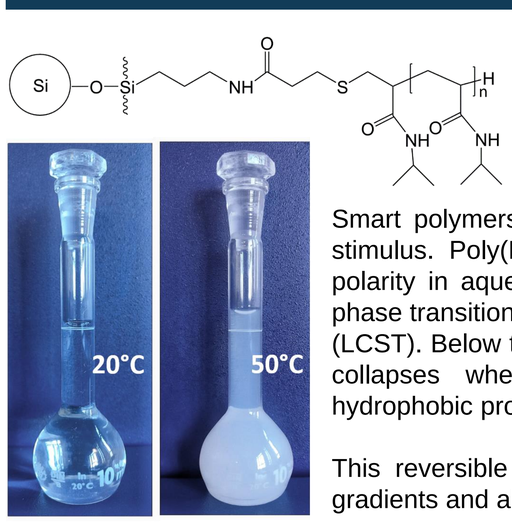
Kristina Wicht[1], Mathijs Baert[1], Sonja Krieger[2], Norwin von Doehren[3], André de Villiers[4] and Frederic Lynen[1]

[1]Separation Science Group, Department of Organic and Macromolecular Chemistry, Ghent University, 9000 Ghent, Belgium
[2] Agilent Technologies, Waldbronn, Germany, [3] Agilent Technologies, Middelburg, Netherlands
[4] Department of Chemistry, University of Stellenbosch, 7602 Matieland, South Africa

INTRODUCTION

Due to the increasing complexity of pharmaceutical drug formulations, assessment of the active pharmaceutical ingredients (API) and of their related impurities is becoming an ever more challenging task by conventional 1D-LC techniques. Baseline separation of all solutes in complex samples can often not be obtained anymore by 1D-LC alone, while the impurities typically arising at very low concentrations require sensitive detection to enable their identification and quantification following current regulations. 2D-LC can offer great benefits especially in regard to peak capacities, but common setups are often facing limitations due to solvent incompatibilities, leading to peak breakthrough, peak broadening and consequently lower sensitivity in the 2D. Only a few comprehensive LCxLC modes, all using purely aqueous separation modes in the first dimension, are exempted from these modulation issues. One of those is the recently introduced combination of temperature-responsive chromatography with RPLC (TRLCxRPLC).[1] Such temperature-responsive phases thereby depict an adaptable hydrophobicity in aqueous solutions, and hence retention as a function of temperature.[2] A main benefit of the approach is that it forgoes the need for organic solvents in the mobile phase. This on the one hand allows for the transfer of high sample volumes from the first to the second dimension with near perfect peak refocussing,[1] and on the other hand facilitates more sensitive detection compared to conventional LCxLC approaches, which often require miniaturization of the first dimension. In this work, the possibilities offered by TRLCxRPLC in terms of sensitivity, peak capacities and quantitative potential are assessed for improved separation of pharmaceutical mixtures. Therefore, synthetic mixtures of pharmaceutical compounds (steroids) are investigated by TRLCxRPLC. Additionally, several column (core-shell) chemistries are assessed in the second dimension (EC-C18, PFP, Phenyl-Hexyl) to evaluate differences in selectivity.

THERMO-RESPONSIVE STATIONARY PHASE



Smart polymers change their properties following to an environmental stimulus. Poly(N-isopropylacrylamide) (PNIPAAm) shows a change in polarity in aqueous solutions, following a change in temperature. This phase transition occurs at the so called lower critical solution temperature (LCST). Below the LCST, PNIPAAm is dissolved in aqueous solution and collapses when the temperature exceeds the LCST, displaying hydrophobic properties (Figure 1).

This reversible phase transition will be used for inverse temperature gradients and allows for the forgo of organic eluents.

Figure 1. Illustration of the temperature-responsive effect of PNIPAAm.

EXPERIMENTAL

	Dimension 1	Dimension 2
Instrumentation	**Agilent 1290 Infinity II 2D-LC System**	
Column	TRLC column PNIPAAm based column 100 x 2.1 mm, 5 µm, 100 Å	Agilent InfinityLab 120 Poroshell 1) EC-C18, 2) Phenyl-Hexyl, 3) PFP 50 x 3 mm, 2.7 µm, 120Å
Flow rate	0.1 ml/min	2.5 ml/min
Temperature	Temperature gradient: 0-30 min: 45ºC 30 min- end: 0ºC	Isocratic 50ºC
Mobile phase	(A) H2O+ 0.1 vol% FA	(A) H2O+ 0.1 vol% FA (B) ACN
Gradient	Isocratic (A)	Figure 2: Segment gradient 0 min - 16.90 min: 25-55% B 17 min - 26.90 min: 30-60% B 27 min - end min: 50-80% B Figure 3: Full gradient 20-80% B
Interface	2-position/8-port valve, tM=1 min, tg= 0.5 min, 120 µl loop	
Detection	DAD at 254 nm	

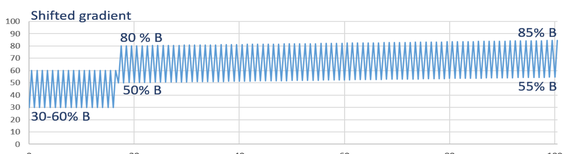
RESULTS AND DISCUSSION

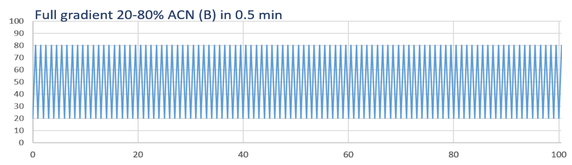
1) ORTHOGONALITY AND PEAK CAPACITIY

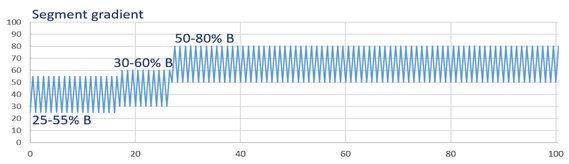
For this work, three different Poroshell columns were tested in the second dimension using 3 gradients to evaluate the ensuing differences in selectivity. The orthogonality was assessed using the Asterisk[3] and the Convex Hull method.[4] Note that also an adapted value was calculated, considering the entire separation space covered by the increasing gradients, and not restricted to the zones delimited by the first and last eluting peaks, as is more “conventionally” the case.

Repeatability was calculated for the retention factor (k) of each peak using the full gradient (TRLCxPFP) for n=3 consecutive measurements. The %RSD (k) for 1D was 0.72% and %RSD (k) in 2D 0.52%. The peak capacity was calculated following Li et al. [5] for the full gradient corrected for undersampling:

$$n'_{c,2D} = \frac{1n_c \times 2n_c}{\sqrt{(1+3.35(\frac{2t_c \times 1n_c}{1t_g})^2)}} = 848$$







	Full gradient				Shifted gradient				Segment gradient			
Orthogonality [%]	Asterisk	adapted	Convex Hull	adapted	Asterisk	adapted	Convex Hull	adapted	Asterisk	adapted	Convex Hull	adapted
EC-C18	36	43	27	15	71	51	52	22	91	49	60	26
Phenyl-Hexyl	40	45	28	13	83	42	53	16	82	49	57	24
PFP	39	44	27	11	91	36	58	12	78	47	55	22

RESULTS AND DISCUSSION

2) SELECTIVITY

Retention on TRLC-based columns at high temperature is based on hydrophobic interactions. In Figure 2 inversed temperature-gradients (from 45° C to 0° C) have been used to elute strongly retained analytes faster. In the second dimension, three Poroshell 120 columns have been used and compared for their selectivity. It can be seen, that the most polar column (PFP) is able to baseline separate compounds 1 & 2 and 15 & 16 more efficiently than the EC-C18 and the Phenyl-Hexyl column, while the EC-C18 column, as the only column, was able to separate an impurity hidden under peak 17. Note, that the gradients have not been optimized for the specific column chemistries.

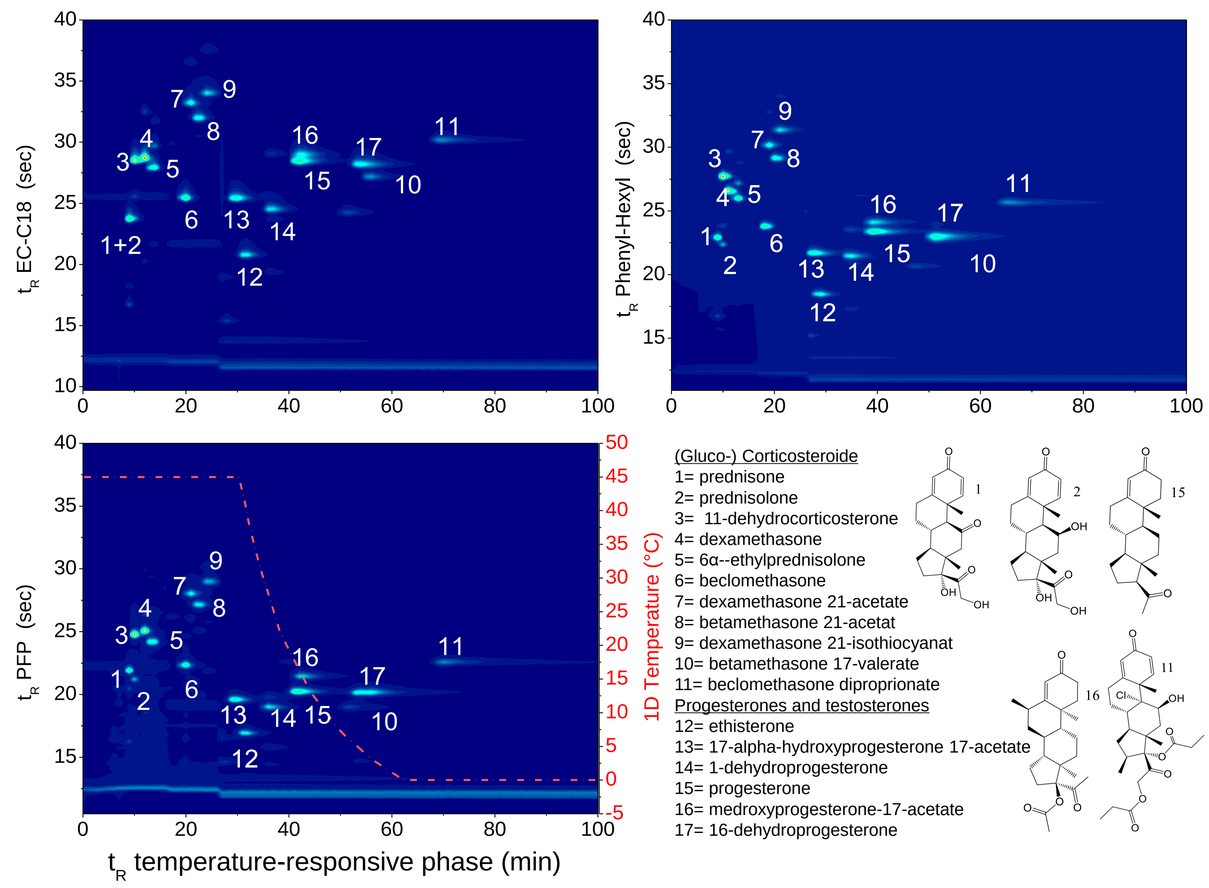


Figure 2. TRLCxRPLC of a steroid mixture (17 compounds) to compare selectivity of 3 different RP-stationary phases in the second dimension for the segment gradient: EC-C18, Phenyl-hexyl and PFP.

3) SENSITIVITY

In Figure 3 an API measurement is shown following ICH guidelines. All Impurities, present at a relative concentration of 0.1% (left) and 0.05% (right) compared to the API were effectively detected in these experiments. In the 0.05% measurement, compounds 7 & 8 are still partially covered by the API, which might be solvable by further lowering the injection volume and adjusting the gradient more specifically. Compounds 2 & 10, though, are more easily visible on the right.

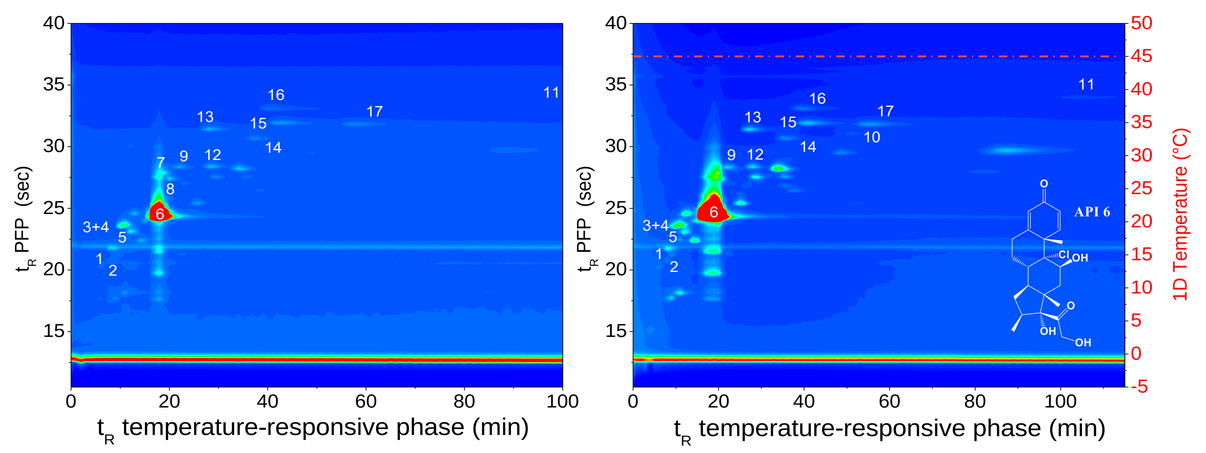


Figure 3. TRLCxRPLC separation of a test mixture of 17 structurally similar steroids at an isocratic temperature of 45ºC and a full gradient in the second dimension: (left) impurities at 2 ppm, API at 2000 ppm, injection volume 6 µl; (right) impurities at 1 ppm, API at 2000 ppm (#6), injection volume 20 µl

CONCLUSION

In this work it was shown, that impurity measurements can be performed with the required sensitivity to detect all 17 compounds. Highest orthogonality was achieved for the combination of TRLCxEC-C18 using the segment gradient. Peak capacities can be further improved by optimizing sampling times.

Future developments

Finding more efficient ways to faster adapt column temperatures for the usage of temperature gradients

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